Chicken model for inducing primordial germ cells differentiating into motile sperms *in vitro*

Hui Xiong[a], Xiangchen Li[b], Qingyun Hu[c*], Pengfei Hu[b] and Weijun Guan[b]

[a]*Histology and Embryology Department, College of Basic Medicine, Jining Medical University, Jining 272000, P.R. China*
[b]*Institute of Animal Science, Chinese Academy of Agricultural Sciences, Beijing 100193, P.R. China*
[c]*College of Basic Medicine, University of Jiamusi, Jiamusi 154007, P.R. China*
**E-mail: jmshuqingyun@163.com*

ABSTRACT

Stem cell technologies have been widely used in the study of spermatogenesis. However, deriving motile sperm from stem cells *in vitro* is still rarely achieved. We found that chicken primordial germ cells could directly differentiate into sperm by using retinoic acid in a non-testicular culture system. The induced sperms were characterised by RT-PCR, immunofluorescence and flow cytometry techniques. Results suggested that chicken primordial germ cells could produce motile sperm *in vitro*. Our work has provided a novel animal model of spermatogenesis *in vitro*, which might be used for male reproductive mechanism research.

Keywords: chicken, primordial germ cells, retinoic acid, induction, motile sperm

1. INTRODUCTION

Spermatogenesis is a complex, multicomponent, interactive process; errors in any link could lead to male oligospermia or azoospermia in clinical practice (Selice *et al*., 2010). The *in vitro* reproductive processes of male gametes has brought new treating strategies for male infertility. *In vitro* sperm-induction from stem cells has made great progress. Various types of stem cells from different animal models have been manipulated, for example, embryonic stem cell (ESC) (Toyooka *et al*., 2003), spermatogonia stem cell (SSC) (Sato *et al*., 2011), induced pluripotent stem cell (iPS) (Hayashi *et al*., 2011), mesenchymal stem cells (MSCs) (Nayernia *et al*., 2006a) and primordial germ cells (PGCs) (Kee *et al*., 2009) while the induction of sperm with motility *in vitro* is still a highly-anticipated issue.

PGCs are the precursor cells of spermatocytes and oocytes (Clawson and Domm, 1969). Initiating the spermatogenesis from PGCs *in vitro* has been reported (Adams and McLaren, 2002; Matoba and Ogura, 2011), but the motile sperm had not been obtained. In our previous study, we have established a PGCs cell line and induced this sort of stem cell to differentiate into cardiomyocytes (Xiong *et al*., 2015), while in this work, motile sperm were derived from PGCs in a culture system outside the testicular environment, and the induced sperm characterised and identified. It is the first description of *in vitro* harvesting motile sperm in an avian species. The chicken genome project has shown that chickens have a similar quantity of gene expression to humans (Callaway, 2014), therefore, we have constructed an excellent animal model for vertebrates on spermatogenesis studies. We have provided an efficient protocol for sperm induction *in vitro*, which could provide abundant operating materials for spermatozoal function research, and could also promote the usage of stem cell transplanting engineering on clinical infertility.

2. MATERIALS AND METHODS

2.1 Experimental animals

Chick and chicken embryos were provided by the Experimental Animal Base Institute of Animal Sciences, Chinese Academy of Agricultural Sciences, Beijing. All experimental procedures involving chick and chicken embryos were conducted in accordance with the guidelines for agricultural animal research codified by the Committee for Ethics of Beijing, P.R. China.

2.2 Cell culture of PGCs

Chicken embryos' gonads (3.5–5.5 d) were isolated using ophthalmic tweezers under a stereoscopic microscope. Samples were dissociated with 0.25% trypsin-0.04% EDTA (Sigma, USA) for 2 min, then neutralised with DMEM containing 10% FBS (Gibco, USA). A 70 μm mesh sieve was used to purify PGCs and cells were centrifuged at 200 g for 12 min at room temperature. Cell deposits

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were resuspended with PGCs culture media consisting of DMEM medium replenished with 10% FBS, 20 ng mL^{-1} of stem cell factor (Sigma, USA), 10 units mL^{-1} of leukaemia inhibitory factor (Sigma, USA) and 20 ng mL^{-1} of basic fibroblast growth factor (Sigma, USA). Suspended cells were seeded at a density of 1×10^4 $well^{-1}$ in the 6-well culture plates which were coated with chicken embryonic fibroblasts (CEFs) as feeder layers (Choi *et al*., 2010). Cultures were placed in a 5% CO_2 and 37.5 °C incubator for primary culture. PGCs colonies were divided and moved into fresh plates with CEFs using trypsin-EDTA treatment for subculture (Liu *et al*., 2013).

2.3 Alkaline phosphatase staining and RT-PCR

An alkaline phosphatase (AKP) staining of PGCs was conducted by an AKP kit (Sidansai, P.R. China). PGCs at passage 3 were collected and the total RNA extracted and then a Reverse Transcription System (Promega, USA) was used. The stage specific genes *CVH*, *CDH* and *Dazl*, the stem cell specific genes *Sox2*, *Pouv* and *Nanog* were detected (primer information is shown in Table 1). The PCR reaction was performed by PCR Master Mix Kit (Promega, USA). PCR products were visualised by electrophoresison 2.5% agarose gels.

2.4 Immunofluorescence staining of PGCs

The stage-specific surface makers of PGCs, SSEA-1 and SSEA-3 (Zhongshan Golden Bridge, Beijing, P.R. China), and the stem cell surface makers *Oct4* and *Sox2* (Zhongshan Golden Bridge, Beijing, China) were detected by immunofluorescence staining following the method of Tsung *et al*. (2003).

2.5 Differentiation potential of PGCs

Embryoid body formation ability was assessed to prove the differentiation potential of PGCs. For a detailed description of this method see Xiong *et al*. (2015). The germ layer specific genes *AFP, GATA6* and *Sox3* were detected by RT-PCR (primer information is shown in Table 1).

2.6 Induction of sperm

The induction medium consisted of PGCs culture medium and RA (10^{-6}mol L^{-1}). PGCs at passage 3 were chosen and reseeded with induction medium into 24-well plates (200 μL per well) at a density of 5×10^4 mL^{-1} in a condition of 5% CO_2 at 37 °C. Fresh induction medium was added every 24 h for nearly a week.

Table 1 Primer information

Gene name	Primer sequences	Temp. (°C)	Product length (bp)	Cycle
AFP	F: TGGGAGACCTGCGGGATA R: TTAAGACTCGGAAGCGAACA	60	436	30
Sox3	F: GGTGGGCCAGAGGATTGA R: CTGCGAGTGCGAGGTGAT	56	328	30
GATA6	F: AGCGCAGAGCTGCTGGAA R: GCAGTTGGCACAGGACAGG	58	213	30
GAPDH	F: TAAAGGCGAGATGGTGAAAG R: ACGCTCCTGGAAGATAGTGAT	60	244	30
CVH	F: GCACAGGTGGTGAACGAA R: TGAATGCTGGTGGATGGT	58	103	30
CDH	F: ATGAAAGCCTTAGGAGTGC R: GAGTGAGGGAGCCAGACA	58	376	30
DAZL	F: AAATGCGGAAGCCCAGTG R: AATTGCGGTGCAGGAGGA	57	393	30
SOX2	F: CAACGGAGGCTATGGGATG R: CGAATGAGACGAGGAGGTGA	59	283	30
POUV	F: GCTCTGGGCACGCTCTAT R: CGTTCCCTTCACGTTGGT	57	231	30
NANOG	F: CGTCCTACGGCTCTGTTA R: CACGTCTTCACCTGCTTG	58	412	30
ACROSIN	F:GGGAAAGGCTGTGGGAGA R:GCAGTCAAGAGGAGGGTGG	57	387	30
PLZF	F:TGGATGACCTGCTCTACGC R:AGTGACTGCCCGATGCTC	57	343	30
TEKT1	F:GCTGGGACTGACTTGGTG R:TAGCTTGTGCTTGGCATC	57	468	30

2.7 RT-PCR assay for sperm-related gene expression

Total RNA of induced sperm was isolated and RT-PCR detection for three sperm-related genes, *ACROSIN*, *PLZF* and *TEKT*1 were conducted (primer information is shown in Table 1).

2.8 Immunofluorescent staining of sperm

The induced sperm were suspension cultured. The general immunohistochemistry was several wash steps, which could lose or damage the spermatids. We used a PerFix-nc Kit (Beckman Coulter, USA) to process immunofluorescence staining. Two sperm-related surface antibodies, Acrosomal and Acrosin, were applied respectively. IgG/FITC (Zhongshan Golden Bridge, Beijing, P.R. China) was added as fluorochrome and DAPI was for nuclei stain. After, cells were observed by a laser confocal microscopy.

2.9 Flow cytometry analysis for the quantity of haploid

The induced spermatozoa were harvested and fixed in 70% ethanol at 4 °C for 12 h, centrifuged and washed by PBS then incubated at room temperature for 15 min in proprium iodide (Sigma, USA) containing RNase A (200 μg mL^{-1}) (Sigma, USA). Induced sperm DNA was analysed by flow cytometry (Beckman Coulter, USA) and the quantity of haploid was calculated, PGCs were took as a contrast.

3. RESULTS

3.1 Characterisation of PGCs

In this study, PGCs were cultured for 5 weeks and cells were treated for subculture for an average of 3 d. A stable PGCs cell line was established which supported the later sperm induction usage (Figure 1A,B). PGCs expressed the stage specific genes *CVH, CDH* and *Dazl,* and the stem cell specific genes *Sox2*, *Pouv* and *Nanog* (Figure 4A). PGCs were easily and strongly positive for AP staining (Figure 1C). PGCs from passage 3 were chosen for the embryoid bodies forming assay (Figure 1D). Embryoid bodies were shown by RT-PCR (primer information is shown in Table 1) (Figure 4B). The immunohistochemistry analysis showed that PGCs expressed the stage-specific surface makers, SSEA-1 and SSEA-3, and the stem cell makers *Oct4* and *Sox2* (Figure 2). These results highlight the unique characteristics of the PGCs cell line.

3.2 Spermatogenesis *in vitro*

During the inducing process, single PGC colonies in suspension status tended to differentiate into sperm. Induction medium was added, then after 12 h, single PGC cells turned into round spermatids with a larger size and a higher refraction. Round spermatids changed to an ellipse shape after 18 h. Spermatids with tails were observed and cells displayed motility. The wagging tail and turning movements were captured by confocal microscopy (see Electronic Supplementary Information, video 1). After 24

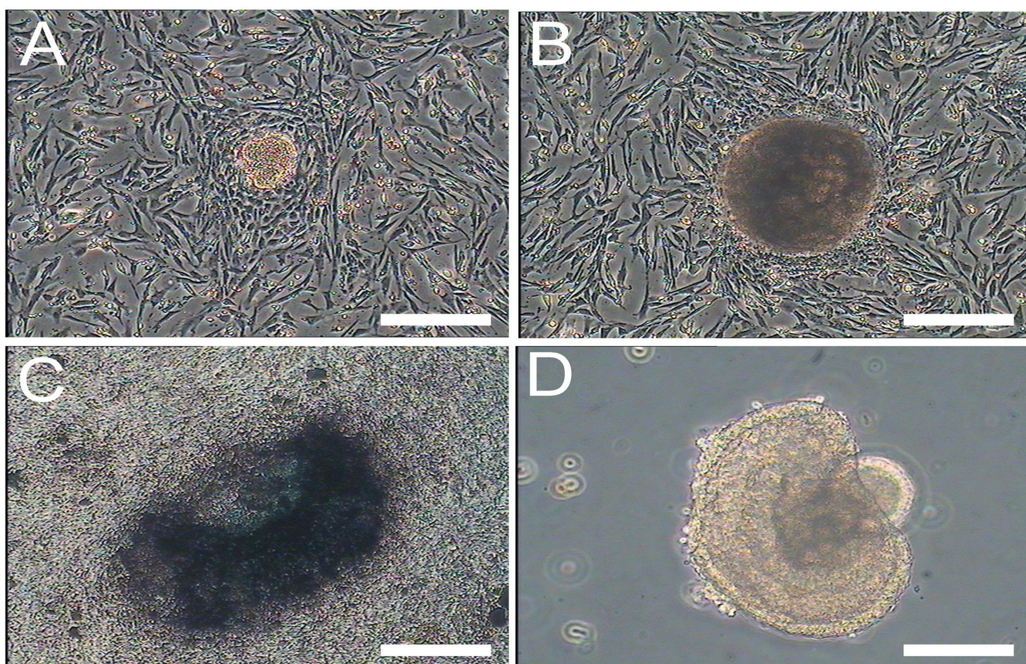

Figure 1 Morphology and AKP staining of PGCs and embryoid body formation. (A) PGCs at passage 3 (scale bar = 50 μm). (B) PGCs at passage 8 (scale bar = 50 μm). (C) Positive AKP staining (scale bar = 25 μm). (D) Suspension embryoid body (scale bar = 50 μm).

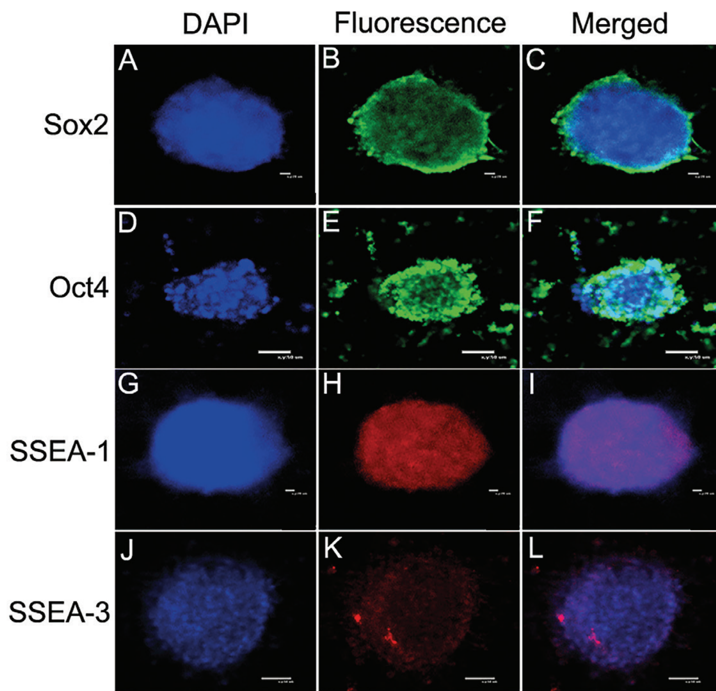

Figure 2 Immunofluorescence of PGCs. (A, B and C) PGC-expressed stem cell specific surface marker *Sox2*, DAPI for nuclei stain, positive fluorescence and merged successively (scale bar = 10 μm). (D, E and F) PGC-expressed stem cell specific surface marker *Oct4* (scale bar = 50 μm). (G, H and I) PGCs-expressed stage-specific embryonic antigen SSEA-1 (scale bar = 10 μm). (J, K and L) PGCs-expressed stage-specific embryonic antigen SSEA-3 (scale bar = 50 μm).

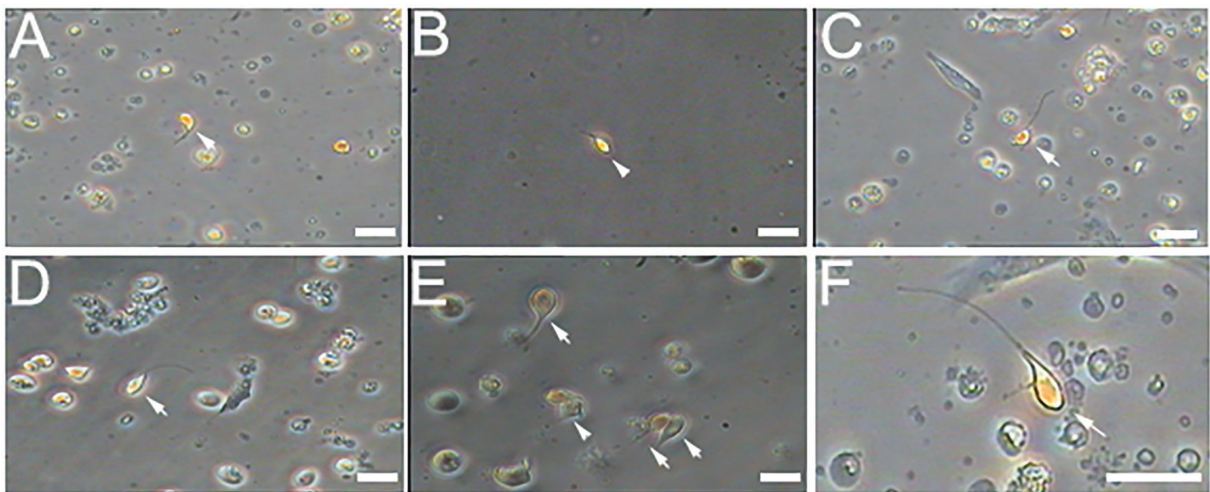

Figure 3 Morphology of induced sperms. (A and B) At 18 h, round spermatids changed to an ellipse shape with a short tail (scale bar = 50 μm). (C) The tail elongated at 24 h (scale bar = 50μm). (D) Tail bending and flapping (scale bar = 50 μm). (E) Sperm number increased at 48 h (scale bar = 50 μm). (F) Mature sperm with clear head, body and tail structure (scale bar = 30 μm).

h, sperm number was increased and tadpole shaped cells became more distinct and remained motile. After 48 h, the structure of mature sperm were formed including head, body and tail. After 72 h (Figure 3A–F), the tail moved faster than before (see Electronic Supplementary Information, video 2), and the motile sperm could move backwards (see Electronic Supplementary Information, video 3) and turn around, but no obvious forward movement was observed. Sperm were continuously cultured for another 3 d.

3.3 Characterisation of induced sperm

We detected the gene expression of induced sperm in three sperm-related genes, where *ACROSIN*, *PLZF* and *TEKT1* were expressed in the transcripts. *ACROSIN* is expressed in the spermatogonia of chickens (Glogowski *et al*., 2001), mice (Brown, 1983) and humans (Keime *et al*., 1990). *PLZF*, named as promyelocytic leukemia zinc finger, is a transcription repressor, which is connected with embryogenesis and the development of male germline stem cells (Costoya *et al*., 2004). *TEKT1* encodes the tubulin of spermatic flagellum and highly affected the motile ability of sperm (Bohring and Krause, 2005). Induced sperm transcribed all the three genes (Figure 4C), which suggests PGCs are involved in spermatogenesis (primer information is shown in Table 1).

We analysed the sperm-related surface markers Acrosomal and Acrosin *via* immunofluorescence staining.

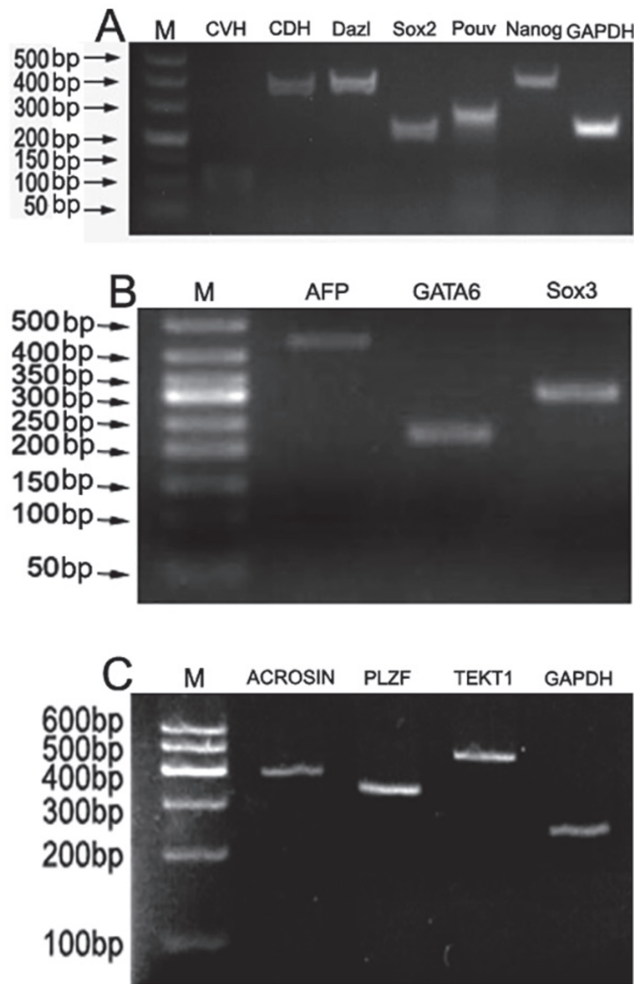

Figure 4 Gene expression of PGCs, embryoid body and induced sperm. (A) PGCs expressed the stage-specific gene *CVH*, *CDH* and *Dazl*, and the stem cell specific genes *Sox2*, *Pouv* and *Nanog*. *GAPDH* served as internal control. All of these bands were the same size as gene products. (B) The embryoid body expressed the genes *AFP*, *GATA6*, *Sox3* from the three germ layers, respectively. (C) The induced sperm expressed three sperm-related genes *ACROSIN*, *PLZF* and *TEKT1*.

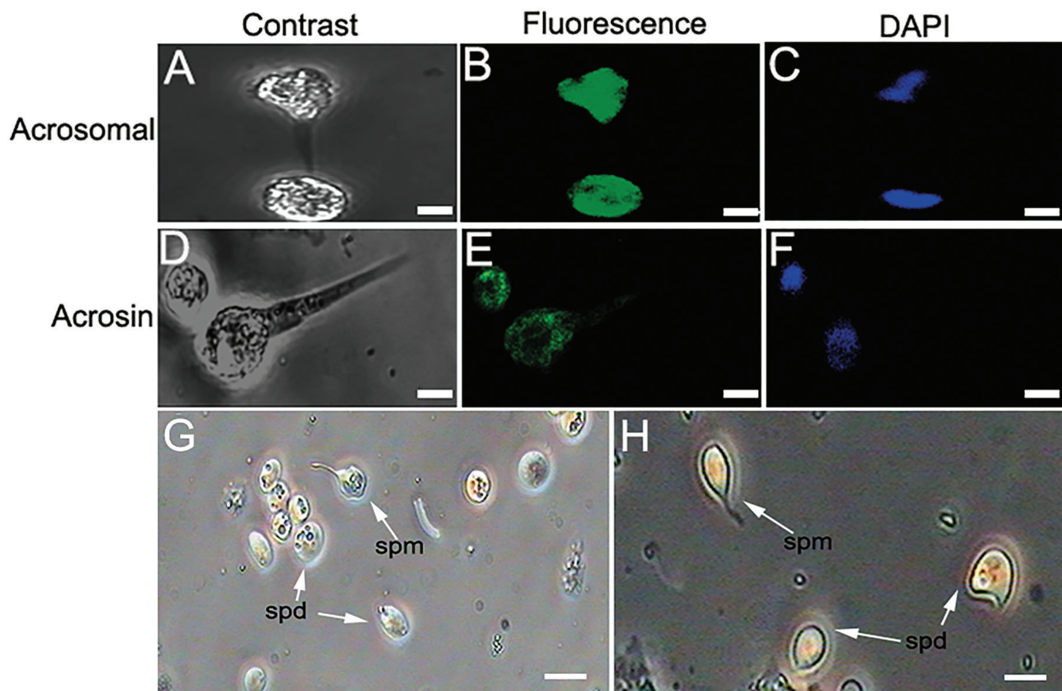

Figure 5 Immunofluorescence staining of induced sperm. (A, B and C) Sperm expressed specific surface marker Acrosomal; contrast, fluorescence and DAPI successively (scale bar = 10 μm). (D, E and F) Sperm expressed specific surface marker Acrosin (scale bar = 10 μm). (G and H) Different development stages of spermatogenesis in morphology, spd indicates round spermatids and spm signifies tailed sperm.

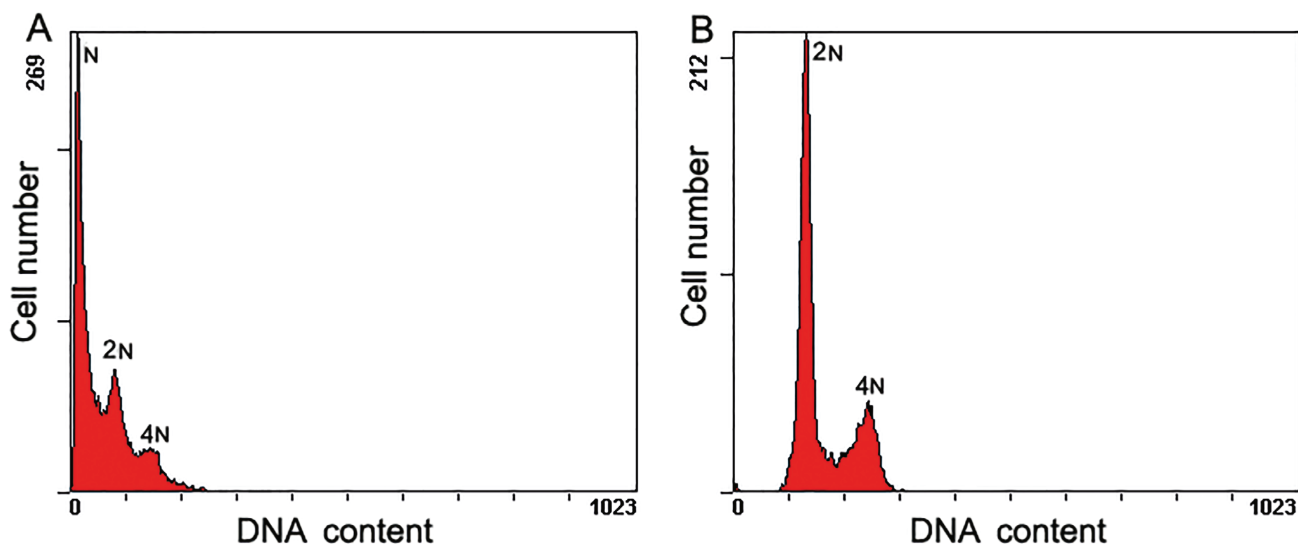

Figure 6 Flow cytometry analysis of haploidy in sperm. (A) Obvious haploid peak of sperm; N is for haploid, 2N is for diploid and 4N is for tetraploid or polyploidy. (B) The typical haploid peak of PGCs.

Acrosomal is essential for the acrosomal enzyme of sperm (Riginos and McDonald, 2003). Spermoblasts expressed these surface makers (Figure 5), thus spermatogenesis was experienced within the manual inductions.

We also estimated the haploid number of induced cells. The haploid peak was prominent and much higher than the diploid or tetraploid peaks (Figure 6A). PGCs were conducted as a control and showed a typical diploid peak without a haploid peak (Figure 6B), which demonstrated that most of the induced cells had undergone a meiosis stage.

4. DISCUSSION

In this study, we successfully obtained motile sperm from chicken PGCs *via* manual induction using RA *in vitro*, which has been rarely reported in deriving male gametes from stem cells in vertebrates. The induced sperm displayed a relatively mature structure and morphology, and exhibited established spermatogenesis phases. The characterisation of induced sperm was also operated.

RA was confirmed to be an effective meiosis inducer. Studies have shown RA affected sexual cells indirectly by regulating the stromal cells, such as sertoli cells or granulosa cells, using multiple-inducers including testosterone and estrogen besides RA to induce stem cell differentiation to male gametes (Kerkis *et al.*, 2007; Nayernia *et al.*, 2006b). In our induction system, there were no gonadal cells but pure PGCs after several generations, suggesting RA might affect germ cells directly.

Temperature is a vital factor during spermatogenesis. A recent report suggested that the temperature during the *in vitro* production of functional sperm *via* testes organ culture was 34 °C (Sato *et al.*, 2011), but birds need a higher culture temperature for their basic body temperature. We cultured the cells in a 37 °C incubator and the induced sperm could survive for 5 to 6 days at this temperature.

Fortunately, we obtained motile sperm *in vitro*, but none of the spermatids could move forwards, meaning the induced sperm were still far from normal standard. The induction efficiency and the mobility of induced sperm should be improved. We have demonstrated a novel model to obtain motile sperm *in vitro* and our current research is focusing on a deeper functional understanding of sperm.

5. ACKNOWLEDGEMENTS

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6. ELECTRONIC SUPPLEMENTARY INFORMATION

ESI is available through stl.publisher.ingentaconnect.com/content/stl/abr/supp-data.

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